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Award Number: W81XWH-11-1-0004

TITLE: Src Kinase: A Novel Target of Early-Stage ER-Negative Breast Cancer

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REPORT DATE: March 2012

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	2. REPORT TYPE	3. DATES COVERED *(From - To)*
01-03-2012	Annual Summary	1 MAR 2011 - 29 FEB 2012

4. TITLE AND SUBTITLE
Src Kinase: A Novel Target of Early-Stage ER-Negative Breast Cancer

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-11-1-0004

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Shalini Jain

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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Texas M.D. Anderson Cancer Center
Houston, TX 77030

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT
Abstract on next page.

15. SUBJECT TERMS
Subject terms on next page.

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	19	19b. TELEPHONE NUMBER *(include area code)*

14.ABSTRACT
Over the last 50 years, the number of cancer related deaths has decreased by only 2%. To achieve reduced breast cancer mortality, it is critical to develop early detection and intervention of breast cancer development at early stages of cancer initiation. This requires better understanding of the critical molecular alterations driving early lesions (atypia) to progress to cancer and developing new interventions based on the observed alterations. Although tamoxifen (Tam) has shown efficacy in prevention of ER+ breast cancers, but they remain ineffective for ER- cancer, therefore new agents are needed to prevent ER- breast cancer. More than 60% of total breast cancer patients are ER-, PR- and HER-2+. However, there are approximately 15% of all types of breast cancer in women who do not express ERα, PR, and HER-2 (5). ER- breast cancer is associated with either HER2-overexpressing (HER2+) luminal-type, or EGFR-overexpressing (EGFR+) basal-type, and other signaling events dysregulating ER pathway. Recently, the crosstalk between the ligand bound ERα and the tyrosine kinase Src has been reported in breast cancer cells, which appears to enhance the proteasomal degradation of ERα. In this study, it has been shown that transfection of Src into MCF-7 cells resulted in decreased levels of ERα protein, but not mRNA. Moreover, they showed that it may be in a subset of ERα- breast cancer with activated Src may lead to the ERα- phenotype. Clinical-pathologic studies have shown elevated c-Src protein levels and/or activity in about 70% of primary human breast and other epithelial tumors, often associated with ErbB2 or EGFR overexpression. Studies from our lab have shown that activation of ErbB2 and downstream signaling pathways can lead to increased Src protein synthesis and decreased Src protein degradation leading to Src up-regulation and activation, which play critical roles in ErbB2-mediated breast cancer invasion and metastasis. Therefore we hypothesized that Src may play important role in the ER- breast cancer initiation and if we can target Src at an early stage the cancer may not progress to ER-. Also targeting Src may reverse the phenotype of ER- DCIS to normal phenotype. In this proposal we will dissect out the in-depth link between Src kinase and ER expression. This study may uncover the novel molecules responsible for ER- cancer initiation which may further help to design effective therapies and strategies of treating ER- breast cancer patients.

15. SUBJECT TERMS
Breast Cancer, ER-, Src

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INTRODUCTION: Over the last 50 years, the number of cancer related deaths has decreased by only 2%. To achieve reduced breast cancer mortality, it is critical to develop early detection and intervention of breast cancer development at early stages of cancer initiation. This requires better understanding of the critical molecular alterations driving early lesions (atypia) to progress to cancer and developing new interventions based on the observed alterations. Although tamoxifen (Tam) has shown efficacy in prevention of ER+ breast cancers (1-3), but they remain ineffective for ER- cancer, therefore new agents are needed to prevent ER- breast cancer. More than 60% of total breast cancer patients are ER-, PR- and ErbB2+. However, there are approximately 15% of all types of breast cancer in women who do not express ERα, PR, and ErbB2 (5). ER- breast cancer is associated with either HER2-overexpressing (HER2+) luminal-type, or EGFR-overexpressing (EGFR+) basal-type, and other signaling events dysregulating ER pathway (4). Recently, the crosstalk between the ligand bound ERα and the tyrosine kinase Src has been reported in breast cancer cells, which appears to enhance the proteasomal degradation of ERα (5). In this study, it has been shown that transfection of Src into MCF-7 cells resulted in decreased levels of ERα protein, but not mRNA (5). Moreover, they showed that it may be in a subset of ERα- breast cancer with activated Src may lead to the ERα- phenotype. Clinical-pathologic studies have shown elevated c-Src protein levels and/or activity in about 70% of primary human breast and other epithelial tumors, often associated with ErbB2 or EGFR overexpression (6-8). Studies from our lab have shown that activation of ErbB2 and downstream signaling pathways can lead to increased Src protein synthesis and decreased Src protein degradation leading to Src up-regulation and activation, which play critical roles in ErbB2-mediated breast cancer invasion and metastasis (9). Therefore we hypothesized that Src may play important role in the ER- breast cancer initiation and if we can target Src at an early stage the cancer may not progress to ER- . Also targeting Src may reverse the phenotype of ER- DCIS to normal phenotype. In this proposal we will dissect out the in-depth link between Src kinase and ER signaling regulation. This study may uncover the novel molecules responsible for ER- cancer initiation which may further help to design effective therapies and strategies of treating ER- breast cancer patients.

BODY:

Aim 1 (Month 6-24): To examine the profile of various signaling molecules by RPPM (Reverse Phase Protein Microarray) those are modulated in the HER2+ ER- DCIS (ductal carcinoma in situ) in mammary epithelial cells 3D culture, MMTV-Neu mouse model and human specimens.

Task 1: To determine the molecules that are altered in HER+ immortalized human mammary epithelial cells.
(a) 1-6 Months: RPPM analysis of the MCF10A vector and MCF10A.ErbB2 overexpressing stable clones.
(b) 6-8 Months: Confirmation of 12 ER- signatures by WB.

Accomplishments: The main goal for this task is to establish the cell line model showing Src activation in ER- cell lines and then perform RPPM analysis and confirmation of the ER- signatures by WB. We achieved this goal by overexpressing ErbB2 in MCF10A and MCF12A human breast immortalized cell lines. ErbB2 overexpressing 10A.B2 and 12A.B2 cells had an increased p-ErbB2 and p-Src (indicating activation) than the 10A.vec and 12A.vec control cell lines respectively **(Figure. 1).** We performed WB/RPPM analysis on these cells cultured in 3D to see if the RPPM signature generated by our previous analysis in patients is represented in the 10A.B2 and 12A.B2 cells. By determining which alterations occur in both (ErbB2-overexpressing mammary epithelial cells) MEC.B2 cell lines, we will gain valuable insight as to what general alterations may be crucial in modulating the key structural alterations of the atypia to DCIS transition (mimicked well in 3D culture). The RPPM performed in 10A.B2 cells showed that ErbB2 overexpression/Src activation drives several molecular changes, importantly the ER- signatures reported in our preliminary human specimen RPPM data **(Figure. 2A&B).** We have submitted the 12A.vec and 12A.B2 lysates for RPPM analysis and waiting for the results to come back. The RPPM data for 10A.B2 cells showed activation of the Akt/Src/P70S6K pathway as we observed in human preliminary data. Moreover, we confirmed

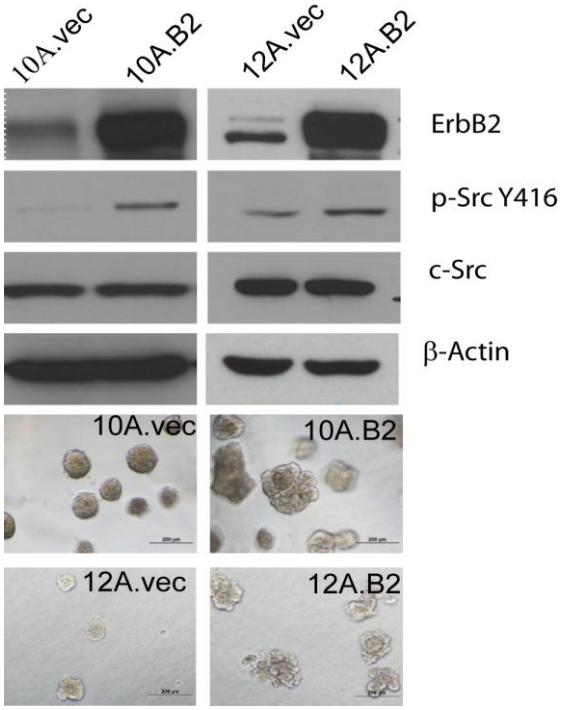

Figure.1 MCF10A and MCF12A cells were overexpressed with either vector or ErbB2 plasmid. Stable clones were established and then 3D cultures were set up using Matrigel. Pictures were taken after 12 days of 3D culture also the expression of Erbb2, p-Src Y416, Src were detected by western blotting and β-Actin was used as loading control.

the ER- signature of ErbB2 overexpressing 10A.B2 cells grown in 3D culture by western blotting. The data showed that ErbB2 overexpression drives Akt/Src/P70S6K pathway activation **(Figure. 3A).** We performed the RPPA analysis in patient specimens also in collaboration with our collaborator at Duke University. The data showed that activation of ER- signature as we had previously reported **(Figure. 2B).**

Justification for Delays/Changes: We have accomplished proposed task within the proposed time line. We have been waiting for our RPPM data in another cell line model, 12A.B2 to come back after processing. The validation of ER- signature in 12A.B2 cells will be done.

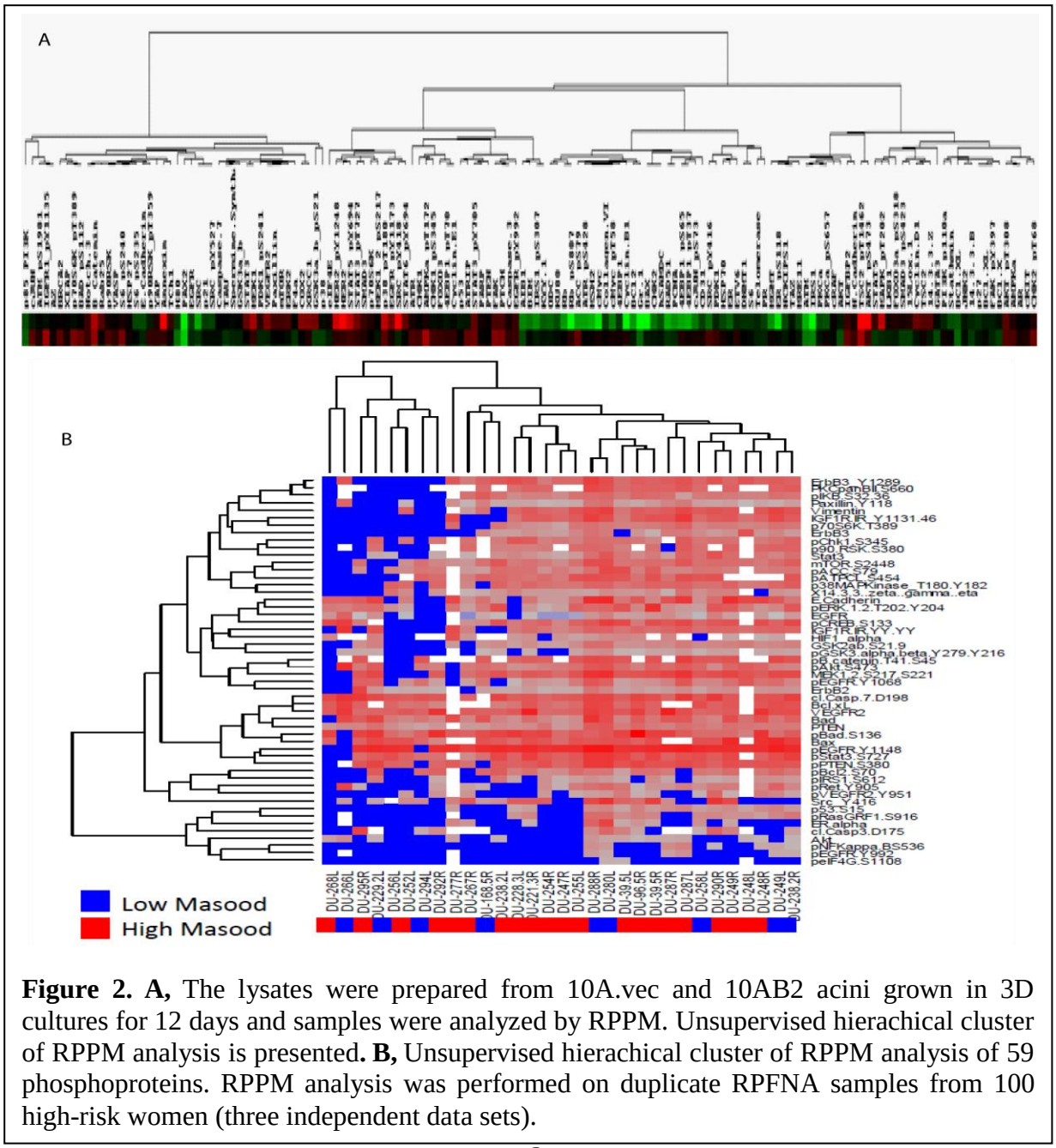

Figure 2. A, The lysates were prepared from 10A.vec and 10AB2 acini grown in 3D cultures for 12 days and samples were analyzed by RPPM. Unsupervised hierachical cluster of RPPM analysis is presented**. B,** Unsupervised hierachical cluster of RPPM analysis of 59 phosphoproteins. RPPM analysis was performed on duplicate RPFNA samples from 100 high-risk women (three independent data sets).

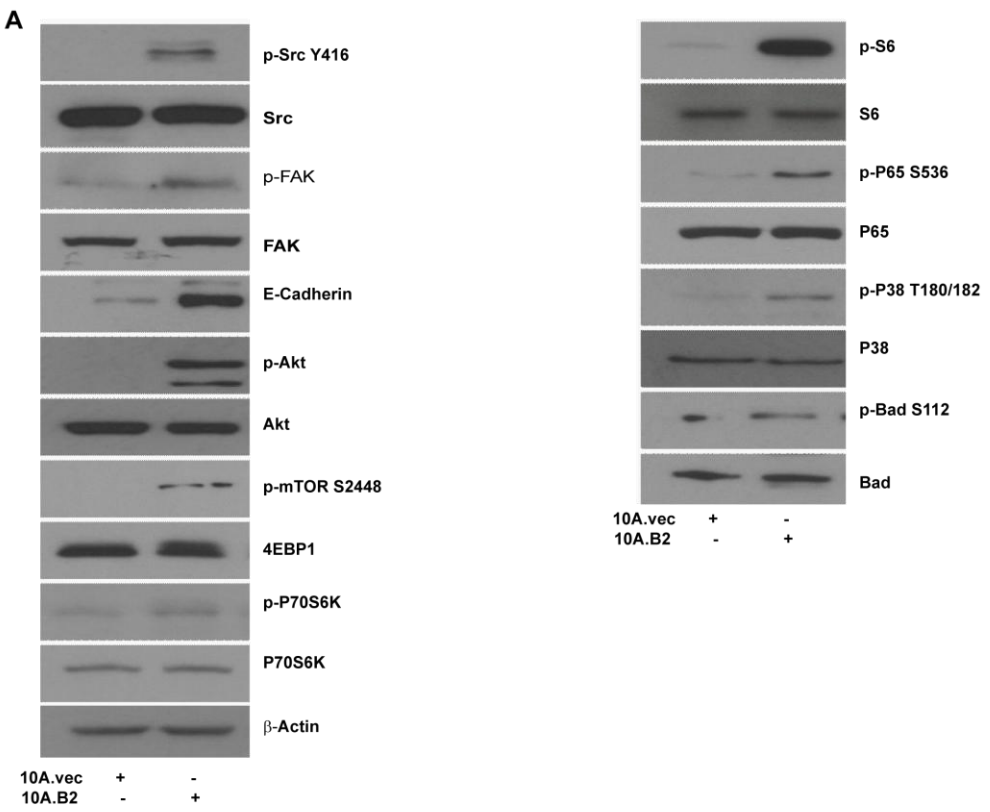

Figure 3. ER- signatures were confirmed by western blot in 10A.vec and 10A.B2 cells grown in 3D culture.

Task 2: To determine the molecules those are altered in MMTV-Neu mouse model.
(a) 18-20 Months: Study the time point of ER loss in MMTV-Neu mouse model.

Justification for Delays/Changes: We have set up breeding of these mice to get Neu+ mice to start these experiments. We will study the time point at which ER is lost in these mice. We will accomplish these tasks within proposed time line.

Task3. To compare the levels of activated Src in ER+ and ER- human breast cancer development.
(a) 12-24 Months: Collect the human breast cancer specimens.
(b) 18-24 Months: Find out the correlation between Src activation and the ER- signatures in these patient specimens by WB and IHC.

Justification for Delays/Changes: We will accomplish these tasks within proposed time line.

Aim 2 (Month 18-30): To determine the role of activated Src in the development of ER- breast cancer.

Task 1: To study the effect of Src on ER status using in vitro model.

(a) 12-30 Months: Effect of Src on ER regulation and acini growth will be identified using MECs, ER+ and ER- cancer cell lines by genetic, molecular manipulations and AZD0530 alone or in combination with Lapatinib.

Accomplishments: To determine the effect of inhibiting Src on ER- signature and acini growth, we set up 3D culture using ER- 10A.B2 cells. The cells were grown in 3D matrigel cultures for initial 6 days and then cells were treated with either vehicle or AZD0530 and the effect of drug on the growth of acini was observed on day 12^{th}. The pictures were taken on day 6^{th} and day 12^{th} of the acini growth. The data showed that AZD0530 prevented the development of acini growth in 3D cultures, whereas cells that were treated with vehicle only formed disorganized (DCIS-like) structure **(Figure. 4A).** These acini were stained with cleaved caspasase-3, Ki-67 and laminin V, p-Src Y416 antibody to characterize apoptosis, proliferation and polarity and p-Src status. The data showed that inhibition of Src induces apoptosis and prevents proliferation in 10A.B2 cells. We sought to determine if AZD0530 suppresses the ER- signature as observed in RPPM analysis, 10AB2 cells 3D cultures were treated with AZD0530 (0.5-1.0 uM) as mentioned in Figure 3A and then the ER- signature were analyzed by western blot. The data showed that inhibition of Src suppresses the ER- molecular signature **(Figure. 4B).** In addition, we were interested to know whether targeting Src down-regulates the metabolic pathway in these acini, we analyzed the expression of PKM2 and LDH-A, two crucial enzymes that are frequently over-expressed in human tumors. Intriguingly, the data showed that inhibition of Src does not affect the expression of PKM2 but inhibits the expression of LDH-A **(Figure. 4B),** an enzyme involved in lactate production in tumors.

Then we were interested to know how long the preventing effect of AZD0530 on the 3D acini growth of 10A.B2 cells can be sustained upon removal of the drug from 3D culture medium. We set up the 3D culture of the 10A.B2 cells for 6 days, after 6 days acini were treated with either vehicle or AZD0530 for another 6 days (like we did in previous experiment), then we washed off the drug from the drug and monitored the acini growth in the absence of the drug for another 12 days. Pictures of the acini were taken after 30 days of culture **(Figure 5A)**. The data showed that even after the drug removal, the acini did not resume their disorganized acini growth and remain smaller like 10A.vec acini. These acini were also stained with proliferation (ki-67), apoptosis (cleaved caspase-3), polarity (Laminin V) markers and p-Src **(Figure 5B).** The data showed that washing off drug from the culture did not resume proliferation, the apoptotic signal was sustained and the p-Src levels did not increased.

Justification for Delays/Changes: We are partly ahead of schedule for completing this task. We will accomplish this task within proposed time line.

Task 2. To investigate the effect of Src on ER status using in vivo model.

(a) 18-20 Months: Study the time point of ER loss in MMTV-Neu mouse model.

(b) 18-30 Months: Investigate the effect of AZD0530 on MMTV-Neu ER- mammary gland tumor development by performing prevention and reversal study.

Justification for Delays/Changes: We will accomplish these tasks within proposed time line.

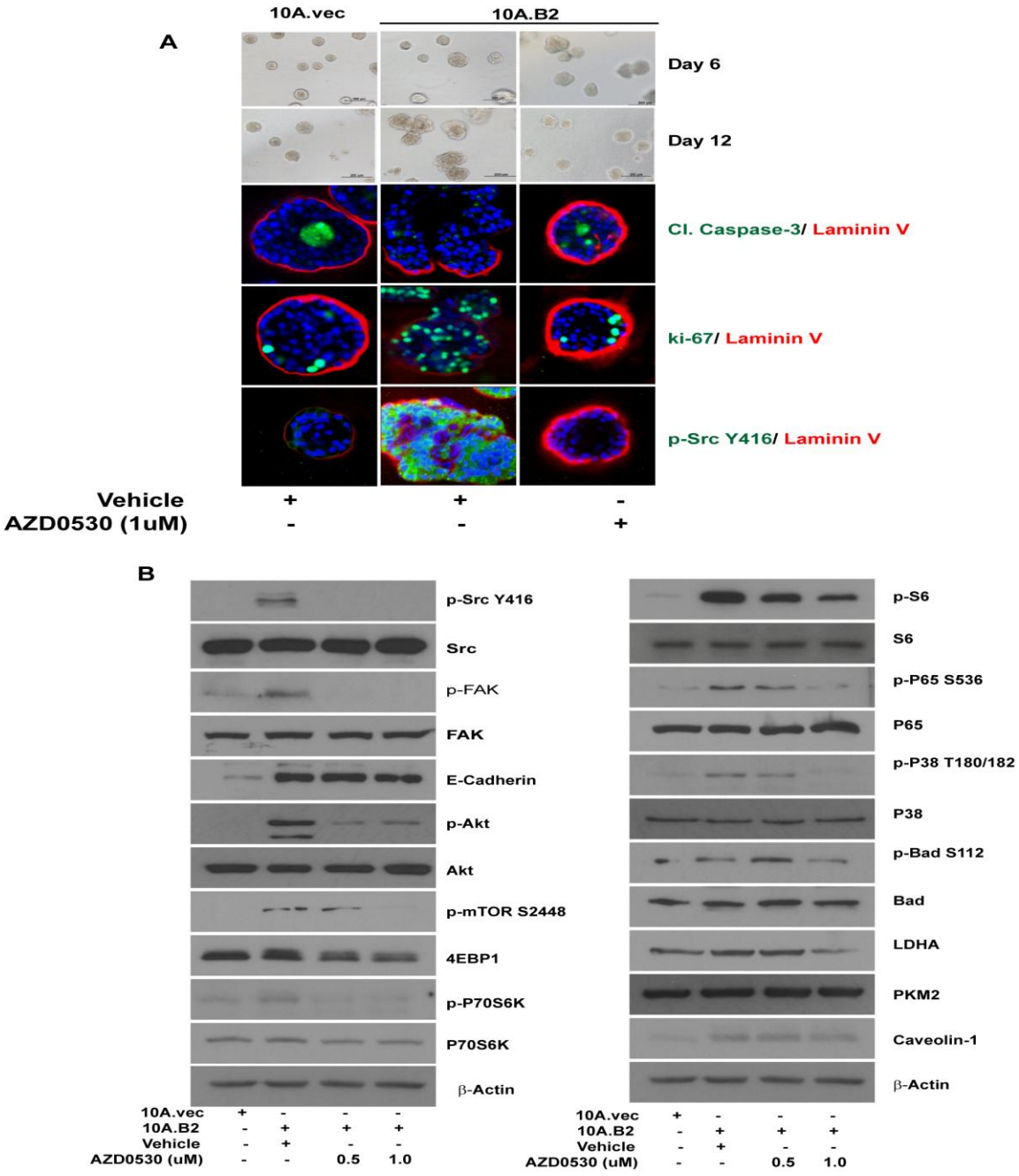

Figure 4. **A,** To examine the effect of AZD0530 on 3D growth of ErbB2- overexpressing 10A.B2 cells, 10A.vec and 10A.B2 cells were grown in 3D culture for 6 days followed by treatment of 10A.B2 cells with AD0530 (1uM) for another 6 days. Pictures were taken on day 6th and day 12th of 3D acini growth. Apoptosis, proliferation and polarity were characterized by immunofluorescence staining using cleaved caspasase-3 (green), Ki-67 (green) and laminin V (red) antibody staining. Src signaling were evaluated by IFS using p-Src Y416 (green) antibody. Blue staining represents DAPI. **B,** The 10A.vec and 10AB2 cells were in 3 D culture and treated with AZD0530 (0.5-1uM) for 6 day as mentioned in panel A, then the cell lysates were prepared and the levels p-Y416-Src, p-Y576-FAK, and E-cadherin), Akt/mTOR signaling (p-S473-Akt, p-mTOR, 4EBP1, p-T412-p70S6K), and p-S536-NFκB, p-p38MAPK, p-S136-Bad were analyzed by western blot.

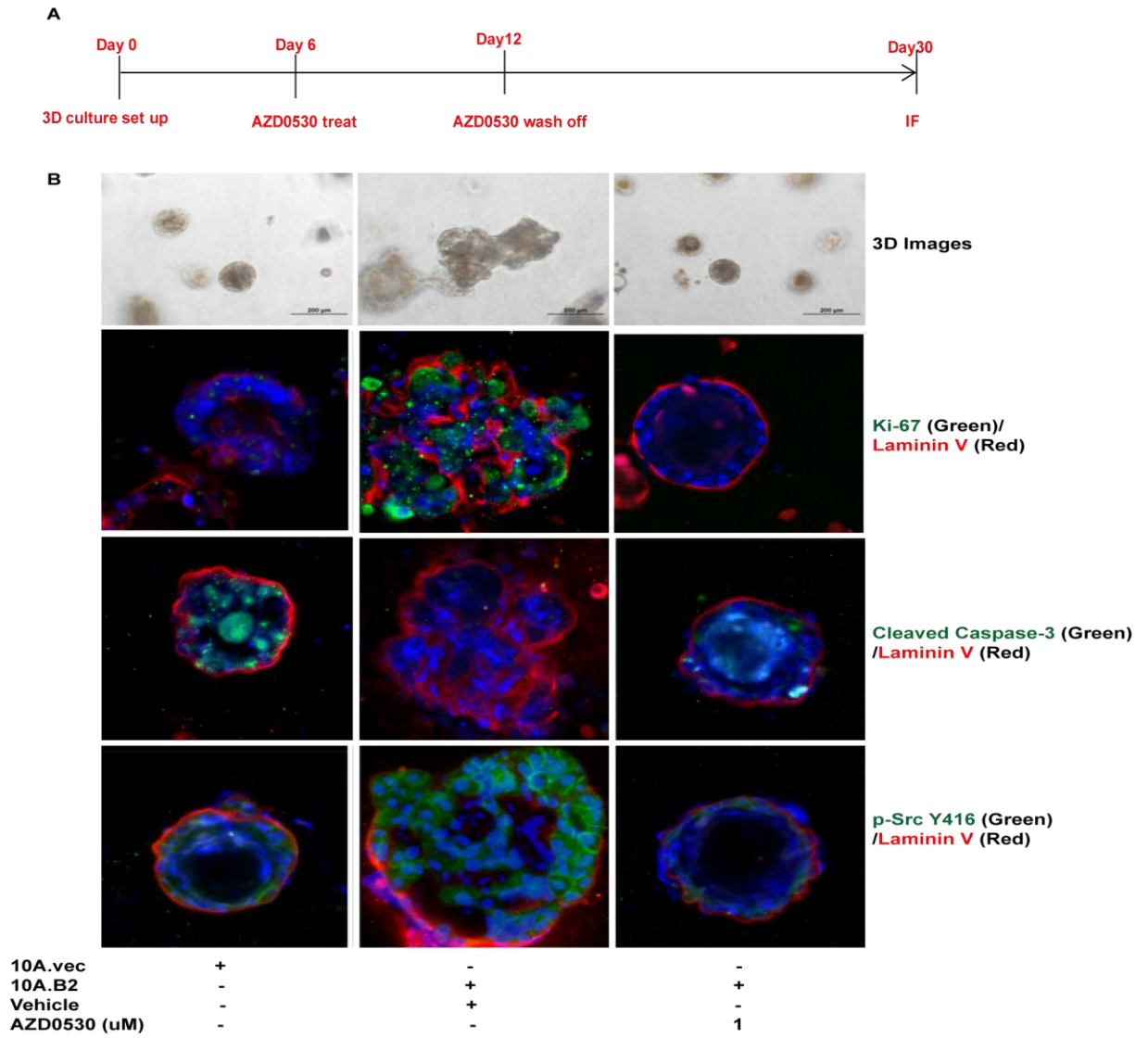

Figure 5. A, Experimental design showing that 3D cultures were set up using 10A.vec and 0AB2 cells. The cells grown initially for 6 days, after 6 days acini were treated with either vehicle or AZD0530 for another 6 days then we washed off the drug from the drug and monitored the acini growth in the absence of the drug for another 12 days. **B,** Pictures of the acini were taken after 30 days of culture. These acini were also stained with proliferation (ki-67), apoptosis (cleaved caspase-3), polarity (Laminin V) markers and p-SrcY416 antibody.

Aim 3 (Month 12-36): To check the effect of targeting Src on the expression levels of ER/PR/ErbB2 in ER- breast cancer and examine the sensitivity of these ER- breast cancer to Tamoxifen/Herceptin using in vitro and MMTV-Neu mouse model.

Task 1. To determine the levels of ER, PR and ErbB2 in ER- breast cancer cells.

(a) 6-12Months: Effect of AZD0530 alone or in combination with Lapatinib will be examined by on the ER, PR, and ErbB2 expression by WB, RT-PCR, and IF in 3D cultures of ER- breast cancer cell lines.

Accomplishments: From the previously proposed study (5) we hypothesized that Src may be responsible for the ER loss/degradation in ER- breast cancer cell line/ER- immortalized human breast epithelial cells. Also, we sought to determine whether Src activation leads to the ErbB2 or PR (Progresterone receptor) loss in ER- breast cell lines. To test our hypothesis we inhibited the Src kinase activity using Src kinase inhibitor, AZD0530 (saracatinib) in the breast cancer cells and MECs. The 10A.B2 cells were treated with either vehicle or AZD0530 (0.5-1.0) in 3D cultures for 6 days. The data showed that AZD0530 does not induce ERα, ErbB2 and PR expression in these cells **(Figure 6A).** The DCIS.com cells were also used if the AZD0530 alone or Lapatinib or the combination of AZD0530 and Lapatinib can reverse the expression of ERα, ErbB2 and PR levels in this cell line. The data indicated that neither of the drug and nor the combination could induce expression of ERα, ErbB2 and PR **(Figure 6B).** Then we analyzed the expression of ERα, ErbB2 and PR in response to the dose dependent AZD0530 treatment in MDA-MB-231 and MDA-MB-435 cell lines. The data showed that targeting/ inhibiting Src does not induce the ERα, ErbB2 and PR expression in these ER- cancer cell line models **(Figure 6C&D).** According to Chu et al, Src promotes estrogen-dependent estrogen receptor α proteolysis in human breast cancer using ER+ or the low ER+ cell lines. Our data in ER- cell lines shows that ER- signature can be reversed using Src inhibitor but not ERα expression as by Chu et al in ER+ breast cancer cell lines.

Justification for Delays/Changes: We have accomplishes this task. The data showed that inhibition of Src in ER- cell lines does not restore the levels of ERα, ErbB2 and PR. Src regulates ERα expression in ER+ cell lines but not ER- cells. We are now interested to find whether inhibiton of Src prevents the development of ER- mammary tumor growth *in vitro* and *in vivo* model and whether targeting Src downregulate the ER- molecular signature and the metabolic pathways in these model systems.

Our data **(Figure. 4A &B)** showed that inhibition of Src can prevent the development of disorganized growth in 3D matrigel cultures, ER- molecular signature. Intriguingly, we observed that targeting Src downregulates LDH-A (Lactate dehydrogenase-A) expression, a critical enzyme involved in lactate production in mammary tumors. Now, we will further analyze the role of Src in regulating LDH-A expression and how it regulates ER- mammary cells growth.

Task 2. Examine the sensitivity of these ER- breast tumors to anti-estrogens.

(1) Time line:

(a) 20-36 Months: To determine the expression of ER expression in these tumors in response to AZD0530, we will perform a time point study to examine the time point at which they restore ER and ErbB2.

(b) 24-36 months: Test the sensitivity of AZD0530 and/Lapatinib treated ER- tumors with Tamoxifen/Herceptin using immunodeficient and MMTV-Neu mouce model. Analyze these tumors and the plasma for breast cancer markers by RPPA, WB, and IHC.

Justification for Delays/Changes: We will accomplish these tasks within proposed time line.

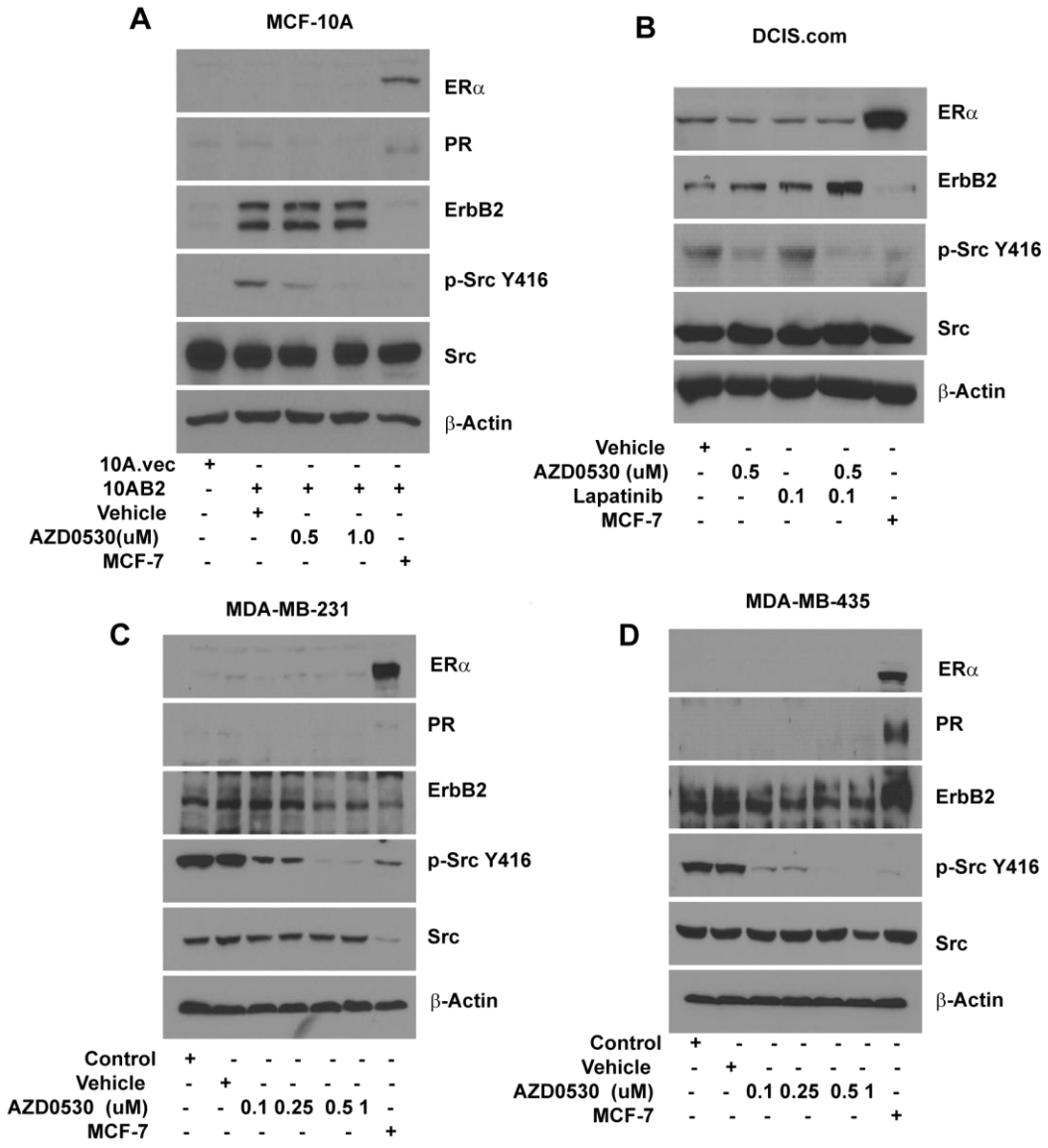

Figure 6. A, 10AB2 cells were treated with either vehicle or AZD0530 (0.5-1.0 uM) for 6 days in 3D culture and the cell lysates were run to analyze the expression level of ERα. PR, ErbB2, p-SrcY416, Src by western blot.. **B,** DCIS.com cells were treated either with vehicle or Lapatinib or AZD0530 or combination of lapatinib and AZD0530 with indicated doses. The levels of **ER**α, ErbB2, p-SrcY416, Src were detected by western blot analysis. **C&D,** MDA-MB-231 human breast cancer cells were either treated with vehicle or the increasing doses of AZD0530 (0.1-1uM). The levels of ERα, PR, ErbB2, p-SrcY416 and Src were analyzed by western blot analysis. The MCF-7 cell lysates was used as positive control. The β-Actin was used as loading control.

KEY RESEARCH ACCOMPLISHMENTS:

- The data showed that the ER- signature as reported in patient specimens exists in ErbB2 overexpressing MCF-10A.B2 cells. We showed it by RPPM and western blot analysis.
- Targeting Src using AZD0530 prevents the development of ErbB2-overexpressing 10AB2 cells disorganized growth in 3D culture condition. We characterized the induction of apoptosis, inhibition of proliferation and polarity using specific markers.
- AZD0530 inhibits the ER- signature in 10AB2 cells.
- Intriguingly, we observed that targeting Src inhibits LDH-A expression, an enzyme responsible for lactate production which serves as an alternative fuel for tumor cells.
- Our data showed that even removal of the drug from the culture medium maintained the preventive effect of Src inhibitor.

REPORTABLE OUTCOMES:

- Poster entitled **"**Src: a Novel Chemopreventive Target for Early Stage ER- Breast Cancer" by Shalini Jain, Maitri Shah and Dihua Yu was presented in 102nd meeting of American Association for Cancer Research, Orlando (Florida), April 2-6,2011.

CONCLUSION:

- Src acts as a node to several RTK dependent downstream cancer cell signaling events, and responsible for turning on the ER- breast cancer molecular signature, that makes Src an important target for preventing the development of ER- breast cancer.
- Cancer cells need more energy than normal cells to proliferate and survive in normal tissue environment. Lactate serves as a good source to provide energy to tumor cells to proliferate and survive in such circumstances. We will further study the role of Src in regulating LDH-A expression *in vitro* and *in vivo* MMTV-neu mouse mammary tumorigenesis model.
- AZD0530 provides long lasting and irreversible prevention in the ErbB2-overexpressing cell line model.
- We will validate our *in vitro* findings in the *in vivo* model, to address whether that inhibiting Src prevents the development of early stage mammary hyperplastic lesions to MIN (mammary intraepithelial neoplasia) lesions.
- However we did not observe that inhibiting Src reverses the ERα, PR and ErbB2 status in ER- cell lines, interestingly our data showed that inhibiting the Src prevents the disorganized growth in 10A.B2 cells by suppressing the ER- signature and importantly it inhibit the crucial LDH-A enzyme that is involved in tumor metabolism.
- In future, targeting Src may be used to prevent the further development of the cancer in patients with early stage ErbB2-overexpressing biopsies and ER- atypia.

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APPENDICES: None

SUPPORTING DATA: None